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Research Paper

A Novel Approach To Investigate the Uptake and Internalization of *Escherichia coli* O157:H7 in Spinach Cultivated in Soil and Hydroponic Medium†

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ABSTRACT

Internalization of *Escherichia coli* O157:H7 into spinach plants through root uptake is a potential route of contamination. A Tn*7*-based plasmid vector was used to insert a green fluorescent protein gene into the *att*Tn*7* site in the *E. coli* chromosome. Three green fluorescent protein–labeled *E. coli* inocula were used: produce outbreak O157:H7 strains RM4407 and RM5279 (inoculum 1), ground beef outbreak O157:H7 strain 86-24h11 (inoculum 2), and commensal strain HS (inoculum 3). These strains were cultivated in fecal slurries and applied at ca. 10^3 or 10^7 CFU/g to pasteurized soils in which baby spinach seedlings were planted. No *E. coli* was recovered by spiral plating from surface-sanitized internal tissues of spinach plants on days 0, 7, 14, 21, and 28. Inoculum 1 survived at significantly higher populations ($P < 0.05$) in the soil than did inoculum 3 after 14, 21, and 28 days, indicating that produce outbreak strains of *E. coli* O157:H7 may be less physiologically stressed in soils than are nonpathogenic *E. coli* isolates. Inoculum 2 applied at ca. 10^7 CFU/ml to hydroponic medium was consistently recovered by spiral plating from the shoot tissues of spinach plants after 14 days (3.73 log CFU per shoot) and 21 days (4.35 log CFU per shoot). Fluorescent *E. coli* cells were microscopically observed in root tissues in 23 (21%) of 108 spinach plants grown in inoculated soils. No internalized *E. coli* was microscopically observed in shoot tissue of plants grown in inoculated soil. These studies do not provide evidence for efficient uptake of *E. coli* O157:H7 from soil to internal plant tissue.

Enterohemorrhagic *Escherichia coli* strains are a leading cause of bloody diarrhea and renal failure in children in industrialized countries. Among enterohemorrhagic *E. coli* strains, those of serotype O157:H7 cause the greatest burden of illness. Contaminated leafy greens have resulted in numerous multistate outbreaks of *E. coli* O157:H7 infection. In 2006, 276 people became ill with *E. coli* O157:H7 associated with the consumption of fresh spinach and shredded lettuce, 148 (54%) were hospitalized, 29 (13%) suffered from hemolytic uremic syndrome, and 3 died *(1, 2)*. These outbreaks were unusual because of the high rates of hospitalization and hemolytic uremic syndrome compared with previous outbreaks *(27)*. The incidence of foodborne outbreaks associated with leafy greens increased by 39% from 1996 to 2005, while leafy green consumption increased by only 9% *(9)*. The principle reservoir for *E. coli* O157:H7 is cattle, and most outbreaks emanate directly or indirectly from this source. Contamination of fresh produce may occur through feces, soil, irrigation water, improperly composted manure, air, wild and domestic animals, equipment, and human handling *(3)*. One potential route of contamination of leafy greens that merits investigation is the uptake and internalization of foodborne pathogens from contaminated soil into edible plant tissue cultivated under realistic preharvest conditions.

Use of the green fluorescent protein (GFP) from *Aequorea victoria* is a powerful method for nondestructive in situ monitoring and visualization of bacteria because GFP expression does not require any substrate addition. Several studies have examined the uptake and internalization of *E. coli* O157:H7 in leafy greens using strains transformed with a *gfp* gene introduced on a plasmid with an ampicillin resistance marker *(10, 13, 26)*. However, such plasmids may affect the fitness of *E. coli* O157:H7 cells *(20)* and their detection in plant tissues. In several studies, transformed *Salmonella* serovars expressing a plasmid-based *gfp* had longer doubling times than did those that did not express this gene *(19)*. This growth disadvantage is of added importance when tracking plant internalization of small in situ populations of bacteria applied to soil and may therefore lead to an underestimation of internalization ability. In the absence of the selective pressure of ampicillin, the bacteria may lose the plasmid and the ability to express *gfp*. Such cells would remain undetected by microscopy in plant tissues or on medium supplemented with ampicillin. In one

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TABLE 1. E. coli *strains and plasmids used*

Strain or plasmid	Genotype or properties	Source or reference(s)
Strains		
RM4407	Wild-type O157:H7 clinical isolate from 2006 outbreak associated with bagged spinach	Robert Mandrell[a]
RM4407-GFP	RM4407 Nal^r *att*Tn*7*::*gfp*	This study
RM5279	Wild-type O157:H7 strain clinical isolate from outbreak associated with bagged vegetables	Robert Mandrell
RM5279-GFP	RM5279 Nal^r *att*Tn*7*::*gfp*	This study
86-24	Wild-type O157:H7 isolated from an outbreak in Walla Walla, WA	*6*
86-24h11	86-24 *stx2* Nal^r	This study
86-24h11-GFP	86-24 *att*Tn*7*::*gfp*	This study
HS	Wild-type commensal strain	*16, 23*
HS-GFP	HS Nal^r *att*Tn*7*::*gfp*	This study
SM10λ*pir*	Host strain containing *mob* loci for plasmid mobilization	*18*
Plasmids		
pTR103	Plasmid containing *mut2* allele of *gfp* under control of a consensus sigma-70 promoter	*7*
pGRG36		*17*
pXLW45	pGRG36 containing *gfp* from pTR103	This study

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such study, the recovery of internalized abiotic fluorospheres from lettuce plants was greater than recovery of an *E. coli* O157:H7 strain expressing *gfp* from a plasmid after 5 days *(25)*. The higher recovery of fluorospheres may be due to the loss of ampicillin resistance (due to plasmid loss) in the *E. coli* O157:H7 in plant tissues; thus, these bacterial would not be detected on selective medium containing ampicillin.

Some studies have assessed *gfp* plasmid stability in *E. coli* O157:H7 strains in vitro but not within plants. Differences in internalized *E. coli* O157:H7 populations containing *gfp* were observed for watercress and spinach plants when bacteria were enumerated on tryptic soy agar (TSA) with ampicillin and sorbitol MacConkey agar without ampicillin *(12)*. However, when *gfp* is inserted into the *E. coli* chromosome, antibiotic selection is not required to maintain the marker. Chromosomal insertion at a suitable location also may mitigate bacterial fitness effects that have been observed in enteric bacteria expressing *gfp*. The objective of this study was to develop strains of *E. coli* O157:H7 and nonpathogenic *E. coli* that contain the *gfp* gene inserted into the bacterial chromosome and then assess the uptake and internalization of these strains into spinach plants (*Spinacia oleracea* L.) during simulated field contamination events.

MATERIALS AND METHODS

Genetic manipulations. The bacterial strains and plasmids used in this study are listed in Table 1. Plates of Luria-Bertani (LB) agar (Becton Dickinson, Sparks, MD) containing 50 μg/ml nalidixic acid were used to select for spontaneous resistance. Strain 86-24h11 is an in-frame *stxA2* deletion mutant of strain 86-24 in which the codons for nine amino acids (FVTVTAEAL) in the active site of the A subunit were replaced with an *Mlu*I restriction site encoding the amino acids T and R as follows. Plasmid pNN11O-18 contains an 842-bp fragment of *stxA2* cloned into the *Pst*I and *Sma*I sites of pUC18. An inverse PCR method was used to generate the 27-bp deletion whereby primers A1 and A2 were constructed complementary to sequences flanking the 27-bp segment encoding amino acids 161 through 169, including the catalytic residue E167. Primer A1 includes an *Mlu*I recognition sequence (underlined) and begins 6 bp downstream of the codon for E167 (5′-CTC ACGCGT GG CGT CGC TTC AGG CAG-3′). Primer A2 also consists of an *Mlu*I recognition sequence (underlined) and is the inverse complement of the codons 18 bp upstream (5′-CTC ACGCCT ACG CAG AAC TGC TCT-3′). PCRs were performed with a Perkin Elmer Cetus (Norwalk, CT) kit and thermocycler for 30 cycles: denaturation for 1 min at 94°C; annealing for 1 min at 50°C, and elongation for 5 min at 72°C. The resulting PCR product included the entire pUC18 and an 812-bp fragment of *stxA2* containing the deletion. This PCR product was digested with *Mlu*I, recircularized with T4 DNA ligase, and amplified in *E. coli* strain DH5α by selecting for ampicillin-resistant colonies. The resulting plasmid containing the 27-bp deleted *stxA2* was called pMW1. An *Sma*I-*Sca*I fragment containing the mutated *stxA2* gene was subcloned into pUC19 to create pMW2 and then further subcloned as an *Sph*I fragment into suicide vector pCVD442 *(5)* to create pAH1. Plasmid pAH1 was introduced into *E. coli* strain 86-24 by conjugation from SM10λ*pir,* and loss of the vector was subsequently selected on sucrose as described *(5)*. Replacement of the wild-type allele with the mutated allele was confirmed by PCR using primers A3 (5′-CGC AGC GCT GGA ACG TTC CGG A-3′) and A4 (5′-TCC ACG TCT CCC GGC GTC ATC G-3′) followed by digestion with *Mlu*I and verified by DNA sequencing. *E. coli* strain 86-24h11 produces immunologically reactive but completely nontoxic Shiga 2 toxoid at levels indistinguishable from those of Shiga toxin 2 produced by the parent strain *(11)*.

For each strain, a nalidixic acid–resistant derivative with a chromosomal insertion of the *gfpmut2* allele *(4)* at the *att*Tn*7* site was selected using previously described methods *(17)*. The *gfpmut2* gene and a consensus sigma-70 promoter were excised from pTR103 with *Not*I and *Xho*I and cloned into pGRG36 to create plasmid pXLW45. Plasmid pXLW45 was mobilized into nalidixic acid–resistant *E. coli* strains from SM10λ*pir,* and exconjugants were selected at 30°C on LB agar containing ampicillin (200 μg/ml) and nalidixic acid. Single colonies were then incubated at 42°C to select for loss of the plasmid, which was verified by loss of ampicillin resistance. Disruption of the *att*Tn*7* site in the chromosome of each strain was confirmed by PCR using the primer pairs Donne-1341 (5′-GAT GAC GGT TTG TCA CAT GGA-3′) and Donne-1342 (5′-GAT GCT GGT GGC GAA GCT GT-3′) *(18)* for strain HS or Donne-1351 (5′-CAA GTG TTC

AAC GGT GCC CC-3′) and Donne-1342 for enterohemorrhagic *E. coli* strains. Donne-1351 and *gfp* primer Donne-1352 (5′-GAC GGG AAC TAC AAG ACA CGT GC-3′) were used to confirm insertion of *gfpmut2* in all strains. PCR conditions consisted of 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 1 min at 68°C with Elongase polymerase (Invitrogen, Carlsbad, CA) and 200 μM concentrations of deoxynucleoside triphosphates. Fluorescent colonies of each strain were confirmed by observation with fluorescent microscopy. GFPmut2 has an excitation peak of 481 nm and an emission peak of 507 nm *(4).*

Preparation of inocula. Three inocula were used to evaluate the ability of *gfp*-transformed *E. coli* to internalize within spinach plant tissues. Inoculum 1 consisted of two modified *E. coli* O157:H7 strains previously isolated from U.S. produce outbreaks: RM4407-GFP (2006 outbreak associated with spinach) and RM5279-GFP (outbreak associated with bagged vegetables). Inoculum 2 consisted of a single *E. coli* O157:H7 strain derived from a strain (86-24h11-GFP) originally isolated from an outbreak associated with ground beef, and inoculum 3 consisted of the nonpathogenic clinical isolate *E. coli* HS-GFP. Strains were individually cultivated on TSA (Becton Dickinson) containing 50 μg/ml nalidixic acid at 37°C for 24 h and then transferred to tryptic soy broth (Becton Dickinson) supplemented with nalidixic acid. Feces obtained from the dairy herd at the U.S. Department of Agriculture Agricultural Research Service (USDA, ARS, Beltsville, MD) were sterilized (at 121°C for 15 min) and then used to make a 1:5 feces:sterile water slurry. The slurry was centrifuged at 10,700 × *g* for 10 min (Allegra 25 R, Beckman Coulter, Fullerton, CA), and the supernatant was harvested and used to culture these strains. All three inocula were adapted for growth in dairy manure by inoculating the individual isolates into the liquid fecal slurry and incubating for 48 h at 37°C at 150 rpm. Inoculated fecal slurries were centrifuged (10,700 × *g* for 10 min), and cell pellets were resuspended and diluted in sterile water and then applied to soils. *E. coli* O157:H7 strains RM4407-GFP and RM5279-GFP (inoculum 1) were grown separately in liquid fecal slurries and combined in equal numbers (as measured by optical density at 600 nm) before inoculation into soil.

Growth of spinach plants. Seeds of the hybrid spinach cultivar 'Avenger', which is resistant to downy mildew lines 1 through 7 and is commonly grown near Salinas, CA, were donated by Seminis, Inc. (Oxnard, CA). Spinach seeds were removed from the package and soaked in a 10% commercial sodium hypochlorite solution for 10 min and the washed three times for 5 min each in sterile deionized water. After washing, 10 to 12 spinach seeds were aseptically placed into sterile seed germination pouches (Mega International, West St. Paul, MN) containing 30 ml of sterile water. Seeds were then germinated for 5 days at 20 to 23°C in the dark. Seedlings were then aseptically transferred to sterile hydroponic growth medium: 10% Hoagland's solution (Sigma, St. Louis, MO) adjusted to pH 5.8 and mixed with 0.2% agar in individual test tubes (16 by 125 mm). Seedlings in the hydroponic medium were then placed in a controlled environment chamber (CMP 4030, Conviron, Winnipeg, Manitoba, Canada) for 7 days under the following conditions: 70 to 72% humidity, light intensity of 1.5×10^{-1} microeinsteins/m^2/s (14 h light, 10 h dark), and 25 ± 1°C. For plants grown in hydroponic medium only, the plants were grown for 7 days before inoculation with *E. coli.*

Preparation of soils. Fine sandy loam (Keyport-Matawan) soil was obtained from the USDA ARS Beltsville area north farm. These soils were steam pasteurized at 85°C for 15 h to kill gram-negative bacteria. Pasteurized soil was placed in sterile "conetainers" (164-ml cone-shaped plastic vessels; model SC10, Stuewe & Sons, Inc., Tangent, OR), each suitable for growing a single spinach plant. During experiments, each plant was irrigated once each week with 20 ml of sterile water supplemented with 1.32 g/liter Jack's Classic All Purpose 20–20–20 fertilizer (J.R. Peters, Inc., Allentown, PA).

TABLE 2. *Populations of* E. coli *in inocula applied to pasteurized soil or hydroponic medium (Hoagland's solution) containing 14-day-old spinach plants*

Growth medium	Inoculum[a]	Population (log CFU/ml)	
		Low	High
Pasteurized soil	1	5.15	8.87
	2	4.65	8.44
	3	5.10	8.79
Hydroponic	1	3.49	6.91
	2	3.17	6.67
	3	4.02	6.9

[a] Inoculum 1, *E. coli* O157:H7 strains RM4407-GFP and RM5279-GFP; inoculum 2, *E. coli* O157:H7 86-24h11-GFP; inoculum 3, *E. coli* HS-GFP.

Inoculation of plant growth medium and soil and microbiological analysis of plants. After 7 days of growth in sterile hydroponic medium, individual seedlings at the two-leaf stage were aseptically transplanted to individual conetainers holding 125 g of pasteurized soil and placed under the same growth conditions as the hydroponic seedlings. After plants were transferred (one plant per conetainer), soils in conetainers were inoculated with 20 ml of inoculum 1, 2, or 3 at either a low (4.65 to 5.15 log CFU/ml) or high (8.44 to 8.87 log CFU/ml) population (Table 2). Inocula were aseptically pipetted onto soil near the spinach shoot tissue, and excess liquid was drained from conetainers. Plants were then placed in the growth chamber under conditions previously described for growth in hydroponic medium. Plants inoculated with sterile liquid fecal slurry containing no *E. coli* served as controls.

Plants also were grown for 21 days in hydroponic medium inoculated with either low or high populations of inoculum 1, 2, or 3 (Table 2). All inocula were prepared as described previously and added to liquid Hoagland's solution (supplemented with 0.2% Bacto agar) and allowed to cool and partially solidify before seedlings were transplanted. Plants were transferred to newly inoculated hydroponic medium every 14 days to maintain initial bacterial levels. After 21 days of overall growth in inoculated hydroponic medium, plants were transferred to uninoculated pasteurized soils under the same growth conditions.

On each day of analysis, three plants grown in soil with low or high populations of inoculum 1, 2, or 3 or in control soil were analyzed. This design resulted in the analysis of 57 plant parts at each time point. Each plant was considered a separate replicate. On days 0 (the day plants were transplanted to soils or into hydroponic medium), 7, 14, 21, and 28, spinach root and shoot tissues were analyzed for internalized *E. coli.* Whole spinach plants (including root tissue) were removed from the soil or hydroponic medium using sterile forceps and immersed in a 5% commercial hypochlorite solution for 10 s and then immersed in sterile deionized water for 5 s to remove soil and debris. Whole plants were then immersed in 0.1% mercury (II) chloride ($HgCl_2$) (Sigma) for 10 min with manual agitation and then in 80% ethanol (Sigma)

for 10 s to kill surface bacteria *(29).* After treatment, plants were rinsed in 1× Dey Engley neutralizing broth (Becton Dickinson) for 10 s and then rinsed twice for 10 s in sterile deionized water. After the excess water was removed, plants were placed in sterile petri dishes (100 by 15 mm; Fisher Scientific, Newark, DE). A sterile no. 11 blade (Feather Safety, Osaka, Japan) was used to dissect plants into shoot tissue (S) and root tissue (R). Root tissue was bisected into R1, the root section immediately adjacent to the shoot tissue, and R2, the root section further away from the shoot tissue and deeper in the soil or hydroponic solution. Each plant part was then placed into 10 ml of sterile 0.1% peptone water in 50-ml conical centrifuge tubes (VWR Scientific, West Chester, PA) and homogenized for 1 min using a Polytron Pt 2100 laboratory blender (Kinematica AG, Littau, Switzerland). The blender probe was sterilized between each sample with 70% ethanol and briefly exposed to an open flame to remove residual ethanol. The probe was then cooled and rinsed using a sterile ice-water bath. Homogenates (0.1 ml, in duplicate) were then spiral plated (WASP2, Don Whitley Scientific, Frederick, MD) on MacConkey agar (Becton Dickinson) supplemented with 50 μg/ml nalidixic acid (MACN) and incubated for 24 h at 37°C. Homogenates also were enriched with 10 ml of 2× EC broth (Becton Dickinson) for 48 h at 37°C. A loopful of each enrichment culture was then streaked on MACN for isolation and confirmation of nalidixic acid–resistant *E. coli.*

Microscopic analysis of plants. Plant parts from three plants grown in soil with low and high populations of inoculum were examined, i.e., 57 plant parts were examined on each day of microscopic analysis. For each plant part, nine fields of view were observed for the presence of internalized fluorescent *E. coli* cells. To observe these cells in spinach tissues, surface-sterilized spinach plants were aseptically dissected into three parts (S, R1, and R2). Each plant part was further sectioned into three subsections and examined using fluorescent microcopy. Wet mount slides were made from thin cross sections of each S subsection and longitudinal sections of each R1 and R2 subsection. All slides were then observed under a light microscope (model E400, Nikon Instruments, Melville, NY) with a fluorescent Intensilight C-HGFI illuminator (Nikon). A Cool Snap HQ camera (Photometrics, Tuscon, AZ) with a charged-coupled device cooled to −30°C was used to visualize fluorescent *E. coli* cells in spinach tissues. Images were digitally recorded using NIS Elements software version 3.0 (Nikon). Spinach was examined microscopically on the same days that samples were collected for cultures (i.e., days 0, 7, 14, 21, and 28).

Microbiological analysis of soil and hydroponic medium. On days 7, 14, 21, and 28, soil that had been inoculated with high populations of each inoculum and then used to grow plants was analyzed for *E. coli.* Twenty-five grams of inoculated soil was placed in 225 ml of 0.1% peptone water in a filtered stomacher bag (Fisher Scientific) and blended in a Stomacher 400 (Seward Ltd., Basingstoke, UK) for 2 min. Appropriate dilutions of blended samples (0.1 ml in duplicate) were spiral plated on MACN. Plates were incubated at 37°C for 24 h, and colonies were enumerated with an automated system (Protocol, Synbiosis, Frederick, MD). Three replicate samples of soil containing each inoculum were taken on each day. Populations of bacteria in the hydroponic medium (Hoagland's solution) in which spinach plants were grown were measured by taking 1 ml of the inoculated medium and serially diluting it in 0.1% peptone water. Samples were spiral plated as described above.

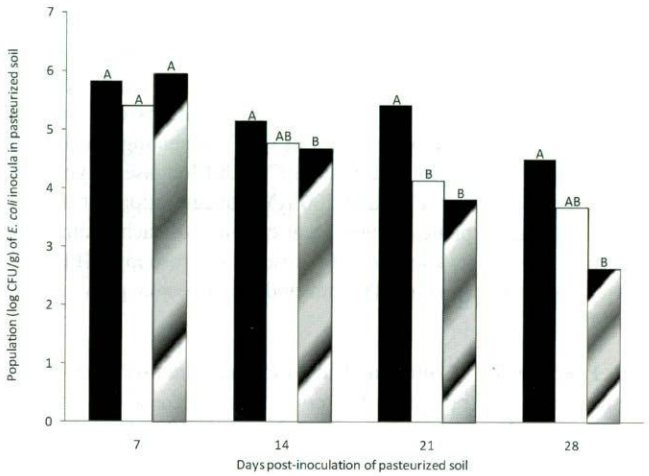

FIGURE 1. *Mean populations (log CFU per gram) of* E. coli *O157:H7 strains RM4407-GFP and RM5279-GFP (inoculum 1, solid bars),* E. coli *O157:H7 strain 86-24h11-GFP (inoculum 2, open bars), and* E. coli *HS-GFP (inoculum 3, hatched bars) in pasteurized soils during 28 days at 25°C. Within each day, different letters above bars indicate significant differences* ($P < 0.05$) *in mean populations* ($n = 3$).

Statistical analysis. Population estimates of each inoculum in soils on days 7, 14, 21, and 28 were subjected to an analysis of variance and Fisher's least significant difference test using Statistical Analysis Software 9.1.2 (SAS Institute, Cary, NC). Results were considered significant at $P < 0.05$.

RESULTS

Recovery of internalized *E. coli* from spinach plants in inoculated soil. *E. coli* was not recovered by spiral plating of samples of spinach plants grown in inoculated soil, regardless of the initial population level (low or high) or which inoculum (1, 2, or 3) was applied to the soil. The detection limit for plate count recovery of inoculum was 50 CFU per plant part (shoot or root). Samples were subjected to an enrichment protocol to determined the presence or absence of *E. coli* when initial values were below the detection limit.

At a low inoculum level, *E. coli* O157:H7 was recovered on day 7 by enrichment of shoot tissue (S) from one of three replicate plants grown in soil containing inoculum 1. On day 28, one of three shoot tissue samples and five of six root tissue samples from plants grown in the presence of inoculum 2 were positive by enrichment for *E. coli* O157:H7. No nalidixic acid–resistant bacteria were recovered at any time point by direct or enrichment culture of any tissue from control plants in uninoculated soil.

When plants were grown in soil with the high populations of the three inocula, similar results were observed. On day 0, one of three enriched shoot tissue samples grown in soil with inoculum 3 was positive by enrichment for *E. coli.* On day 28, one of six enriched root tissue samples grown in soil with inoculum 1 yielded *E. coli* O157:H7.

E. coli inoculated at high populations into pasteurized soil were enumerated to compare the survival of the three inocula on days 7, 14, 21, and 28 (Fig. 1). Significant dif-

TABLE 3. *Populations of* E. coli *recovered by direct spiral plating on MACN and after enrichment from surface-sanitized spinach plant sections exposed to an initial* E. coli *population of ca. 4 log CFU/ml in hydroponic medium*

		Recovery of *E. coli*[c]							
		Day 7		Day 14		Day 21		Day 28	
Inoculum[a]	Plant section[b]	log CFU	Enrichment	log CFU	Enrichment	log CFU	Enrichment	log CFU	Enrichment
1	S	ND[d]	0	ND	0	4.35[e]	1	ND	0
	R1	ND	0	ND	0	3.03[f]	2	ND	0
	R2	ND	0	ND	0	2.69[e]	2	ND	0
2	S	ND	0	2.00[f]	2	1.7[f]	1	ND	0
	R1	ND	0	ND	0	ND	0	ND	0
	R2	ND	0	3.20[f]	1	2.57[f]	1	ND	0
3	S	ND	0	2.00[f]	2	2.00[f]	2	1.88[f]	1
	R1	ND	0	ND	1	ND	1	ND	0
	R2	ND	0	2.51[f]	2	4.67[f]	1	ND	1

[a] See Table 1 for strain composition of each inoculum.
[b] S, shoot (above ground tissue); R1, upper root section; R2, lower root section.
[c] Recovery by plating expressed as log CFU per shoot or root. Recovery by enrichment expressed as number of positive samples of three enriched samples.
[d] ND, populations were below the detection limit of 1.7 log CFU per shoot or root.
[e] Values based on recovery from only two of the three replicates.
[f] Values based on recovery from only one of the three replicates.

ferences were observed between inoculum 1 and inoculum 2 populations on day 21, and between inoculum 1 and inoculum 3 populations on days 14, 21, and 28. Low populations of inocula 1, 2, and 3 in soil declined by 1.48, 2.65, and 2.28 log CFU/g, respectively, between day 7 and day 21 before becoming undetectable by spiral plating on day 28.

Recovery of internalized *E. coli* in spinach plants grown in inoculated hydroponic medium. *E. coli* was internalized sporadically into tissues of spinach plants grown in hydroponic medium inoculated with low bacterial populations (Table 3). On day 7, spiral plating and enrichment resulted in no recovery of internalized *E. coli* from plants grown in hydroponic medium. *E. coli* was recovered on day 21 more frequently than on any other day of analysis from plants infected with inoculum 1. After plants were transferred to uninoculated soil on day 21, *E. coli* from only inoculum 3 was recovered from one of three replicate shoot tissue samples taken on day 28.

Similarly, no internalized bacteria were recovered on day 7 from spinach plants grown in hydroponic medium

TABLE 4. *Populations of* E. coli *recovered by direct spiral plating on MACN and after enrichment from surface-sanitized spinach plant sections exposed to an initial* E. coli *population of ca. 7 log CFU/ml in hydroponic medium*

		Recovery of *E. coli*[c]							
		Day 7		Day 14		Day 21		Day 28	
Inoculum[a]	Plant section[b]	log CFU	Enrichment	log CFU	Enrichment	log CFU	Enrichment	log CFU	Enrichment
1	S	ND[d]	0	ND	1	4.43[e]	1	ND	0
	R1	ND	0	ND	0	ND	0	ND	0
	R2	ND	0	2.67[e]	1	2.99[e]	1	ND	0
2	S	ND	0	3.73[f]	3	4.35[f]	3	3.12[e]	1
	R1	ND	0	2.35[e]	0	1.39[e]	1	ND	1
	R2	ND	0	3.20[e]	1	2.10[e]	1	2.17[e]	1
3	S	ND	0	ND	1	3.45[g]	3	1.88[e]	1
	R1	ND	0	ND	0	ND	1	ND	0
	R2	ND	0	1.39[e]	1	1.88[e]	2	2.79[e]	1

[a] See Table 1 for strain composition of each inoculum.
[b] S, shoot (above ground tissue); R1, upper root section; R2, lower root section.
[c] Recovery by plating expressed as log CFU per shoot or root. Recovery by enrichment expressed as number of positive samples out of three enriched samples.
[d] ND, populations were below the detection limit of 1.7 log CFU per shoot or root.
[e] Values based on recovery from only one of the three replicates.
[f] Values based on recovery from all three of the three replicates.
[g] Value based on recovery from only two of the three replicates.

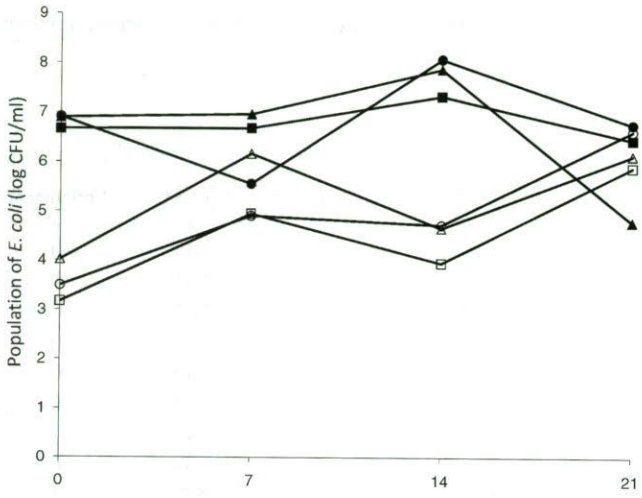

FIGURE 2. *Populations of* E. coli *inocula (log CFU per milliliter) recovered from hydroponic medium (Hoagland's solution with 0.2% agar) in which spinach plants were grown at 25°C for 21 days. On day 14, spinach plants were transferred to medium freshly inoculated at day 0 low or high target populations. Low populations for inocula 1 (○), 2 (□), and 3 (△) and high populations for inocula 1 (●), 2 (■), and 3 (▲) are mean values (*n *= 3).*

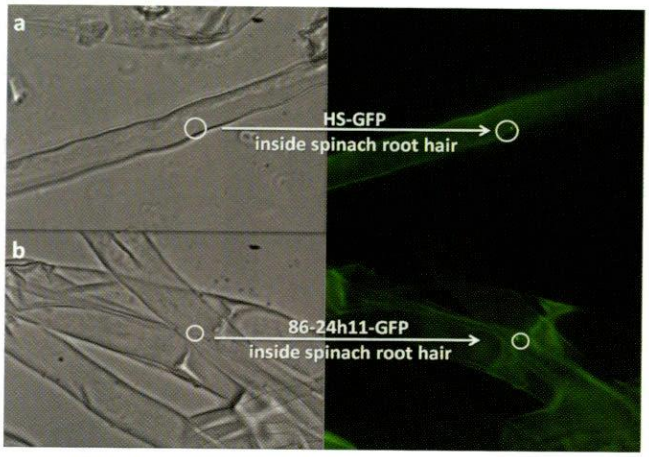

FIGURE 3. *Detection of (a)* E. coli *HS-GFP in a root hair of a spinach plant grown in inoculated soil for 7 days and (b)* E. coli *O157:H7 86-24h11-GFP in a root hair of a spinach plant grown in inoculated soil for 14 days. Brightfield images (left) were obtained with an oil-immersion phase contrast 100× objective, and fluorescent images (right) were obtained with a 100× Plan-fluor oil immersion objective and a fluorescent illuminator to excite GFPmut2 at 481 nm (emission peak at 507 nm). Autofluorescence of the plant tissue is visible but can be differentiated from the brighter emissions of the fluorescent* E. coli. *All images were digitally recorded using a Cool Snap HQ camera with a charged-coupled device cooled to −30°C.*

containing high populations of *E. coli* (Table 4). For plants grown in the presence of inoculum 2, all three replicate shoot samples yielded *E. coli* by direct spiral plating on days 14 and 21, with mean populations of 3.7 and 4.35 log CFU per shoot, respectively. On day 21, two of three replicate shoot tissue samples from plants grown in the presence of inoculum 3 were positive by direct spiral plating, with a mean of 3.45 log CFU per shoot for the two samples. However, once plants were transferred to pasteurized uninoculated soil, viable *E. coli* was recovered from only a single shoot sample from a plant grown in the presence of inoculum 2 (3.12 log CFU per shoot).

The populations of *E. coli* in hydroponic medium at 25°C increased at least 1.4 log CFU/ml from day 0 to day 7 after application of all three inocula at low populations (Fig. 2). Similarly, after plants were transplanted on day 14 into hydroponic medium freshly inoculated with low populations of *E. coli,* all populations increased by a similar amount. However, medium initially inoculated with high populations of inocula 1, 2, and 3 remained static or decreased from day 0 to day 7 and also decreased from day 14 to day 21.

Microscopic analysis. Fluorescent *E. coli* cells were not detected by microscopy in any root or shoot samples from spinach plants grown in soil containing the low-population inocula. No *E. coli* cells from any inoculum, regardless of initial population (low or high), were observed in shoot tissues on any day. Fluorescent *E. coli* cells were observed sporadically in root tissues when plants were grown in soils and hydroponic medium with high-population inocula (Fig. 3). Fluorescent *E. coli* O157:H7 cells were visible on day 7 in 14 (78%) of 18 fields of view of root subsections grown in soils with high populations of inoculum 1. For root subsections grown in soils with high populations of inocula 2 and 3, 4 (22%) of 18 and 5 (28%) of 18 fields of view contained internalized fluorescent *E. coli* O157:H7 cells, respectively, on day 7. On day 14, 3 (17%) of 18 fields of view of root subsections grown in soil with high populations of inoculum 1 and 4 (22%) of 18 fields of view of root subsections from inoculum 2 had fluorescent *E. coli* cells. No fluorescent *E. coli* cells were observed in tissues of spinach plants grown in uninoculated soil.

DISCUSSION

Enterohemorrhagic *E. coli* strains belonging to the O157:H7 serotype cause outbreaks of hemorrhagic colitis that have severe and sometimes fatal consequences. To prevent disability and death from this highly virulent pathogen, we must understand the routes and mechanisms by which produce becomes contaminated. One mechanism that has received recent attention is the possible internalization of *E. coli* O157:H7 into tissues of intact plants, because internalized bacteria would be impossible to eradicate by washing. Previous attempts to determine whether *E. coli* O157:H7 can enter tissues of intact plants have involved bacteria carrying plasmids that provide selective markers or GFP expression. The work presented here represents the first use of *E. coli* with a chromosomal GFP insertion for a study of uptake and internalization in leafy greens. The insertion of *gfpmut2* at the *att*Tn*7* site does not require antibiotic selection, disrupt the expression of other genes, or decrease fitness of the bacteria *(17, 21, 22).* The *att*Tn*7* insertion site is in a highly conserved region of the noncoding 3′ end of the *glmS* gene, a gene essential for the synthesis of *N*-acetyl-glucosamine in *E. coli* and other

gram-negative bacteria *(21)*. The use of the strains constructed in our study provided for sensitive detection of internalized bacteria in spinach tissues without compromising the bacteria. Using these strains, we found only limited evidence of internalization to spinach plants despite continuous exposure to high populations of bacteria in the growth medium.

In previous studies, various surface sterilization methods have been used to examine the uptake and internalization of *E. coli* O157:H7 and *Salmonella* into leafy green tissues *(10, 12, 13, 25)*. Our preliminary work (data not shown) for this study revealed that 0.1% mercury chloride (10 min) plus 80% ethanol (10 s) followed by two successive washes in deionized water (10 s) was more effective for killing surface bacteria than was 0.1 % silver nitrate (10 s) *(8, 29)*.

The three inocula used in this study were evaluated for their ability to internalize into spinach tissue and persist in soils. The inocula were formulated based on their source: isolates from outbreaks associated with produce (inoculum 1), an isolate from an outbreak associated with ground beef (inoculum 2), and a clinical commensal isolate (inoculum 3). Populations of all *E. coli* inocula declined in pasteurized soils, based on direct plating on MACN. However, populations of inoculum 1 (*E. coli* O157:H7 strains RM4407-GFP and RM529-GFP) were significantly higher than those of inoculum 3 (*E. coli* HS-GFP) on days 14, 21, and 28 (Fig. 1). The use of two strains in inoculum 1 and only one strain each in inoculum 2 and inoculum 3 may partially account for differences in survival observed in these pasteurized soils. However, the enhanced survival of inoculum 1 compared with inocula 2 and 3 may have resulted in more frequent observation of these cells in root tissue on day 14. It was not possible to differentiate the ability of these strains to internalize into spinach tissues because no internalized populations were recovered from spinach plants grown in the inoculated soils.

The steam treatment of these soils (85°C for 15 h) reduced the number of gram-negative microorganisms present and may have influenced the survival of inoculated *E. coli* in these soils. Soils were steam pasteurized to avoid the potential production of reduced forms of iron, manganese, and other microelements that can impart toxicity to the substrate when composts or soils are autoclaved *(28)*. The pasteurization of these soils may have eliminated a subpopulation of gram-negative microbial competitors of *E. coli* O157:H7, which could potentially internalize into spinach root tissues in soil. Even with this potential competitive advantage given to both low and high populations of *E. coli* in soil, none of the inoculum strains were able to translocate to root or shoot vascular tissue. The physiological stress on *E. coli* O157:H7 in soil may affect the ability of these cells to internalize into spinach tissue.

Populations of inoculum 2 at 3.7 and 4.35 log CFU per shoot were recovered in shoot tissue from all three replicate plants grown in inoculated hydroponic medium (semisolid medium of Hoagland's solution with 0.2% agar) on days 14 and 21. It is unclear why *E. coli* populations in this medium were able to internalize to spinach shoot tissue but populations in soils were not. The semisolid nature of the medium may have allowed motile *E. coli* cells to traverse through the medium and encounter the root hairs. Low initial populations in the hydroponic medium grew by 1.42 to 2.14 log CFU/ml between day 0 and day 7, when plants were transplanted to freshly inoculated medium. In contrast, low populations of *E. coli* in soil declined to less than 1 log CFU/g by day 21 (data not shown), indicating that cells were under less physiological stress in hydroponic medium than in pasteurized soils. Increasing Matric potentials in soils could cause physiological stress, especially to enteric pathogens not adapted to a rhizospheric environment *(24)*. Such stresses are clearly absent in hydroponic medium. The lack of physiological stress on *E. coli* cells in the hydroponic medium therefore improved the ability of these cells to internalize into spinach tissues. Why internalized populations of inoculum 2 were recovered consistently from shoot tissue and not root tissue on days 14 and 21 is not clear. Internalization of foodborne pathogens may be more frequent in hydroponic systems because there is less competition for nutrients from rhizospheric bacteria abundant in soil, and pathogens in these systems may have physiological fitness advantages when colonizing root tissue, providing a platform for internalization *(14)*.

Other researchers have examined uptake and internalization of enteric pathogens into leafy greens grown in a hydroponic medium. *E. coli* O157:H7 did not internalize to root tissue of lettuce seedlings grown hydroponically but *Salmonella* Typhimurium did *(9)*. Differences between these results and our data may be due the different formulations of the hydroponic media used. Our experiments incorporated 0.2% agar into Hoagland's solution, possibly bringing *E. coli* cells into more intimate contact with spinach roots and providing better conditions for uptake. Three *Salmonella* serovars inoculated into 0.5% Hoagland's agar were able to internalize to lettuce seedlings after 3 or 6 weeks of growth *(14, 15)*. However, only one of these serovars (*Salmonella* Dublin) was able to internalize to lettuce seedlings when grown in inoculated soil. These results support the hypothesis that semisolid Hoagland's agar may provide a better platform than soil for *E. coli* to enter into roots of leafy green plants.

Microscopic analysis revealed fluorescent *E. coli* cells of inocula 1, 2, and 3 in root hairs after 7 days of growth in soils inoculated with high populations (Fig. 3). However, differences in populations of soil inocula were not significant at this stage (Fig. 1), indicating that these cells may not be as physiologically stressed as on days 14, 21, or 28. However, no cells of any inoculum were observed in root subsections on day 21 or day 28. The lack of fluorescent *E. coli* cells in root tissues on days 21 and 28 indicates that as physiological stress on *E. coli* increased in soils, cells were less likely to internalize into intact root tissues. An absence of physiological stress on *E. coli* cells may be required for cells to internalize into intact root tissue in soils. No *E. coli* cells were microscopically observed in shoot tissues from plants grown in inoculated soil. The absence of *E. coli* O157:H7 from larger observed sections suggests that the inherent anatomical and physiological barriers in

intact plant tissues prevent *E. coli* O157:H7 from internalizing through uptake.

We used chromosomally transformed *gfp–E. coli* strains to evaluate the uptake and internalization of *E. coli* into spinach tissues. Internalization did not occur for root or shoot tissue when plants were grown in soils inoculated with *E. coli* at 5 or 8 log CFU/ml. However, internalization of *E. coli* did occur infrequently in shoot tissue after at least 14 days of growth and only in hydroponic medium inoculated with *E. coli* at ca. 7 log CFU/ml. This sporadic and low level of internalization does not appear to be an exclusive property of *E. coli* O157:H7 strains; sporadic internalization of a commensal *E. coli* strain also was observed. Internalization of *E. coli* O157:H7 from contaminated soil to spinach plants appears unlikely. More evaluation is needed to truly define a mechanism by which contaminated hydroponic medium provides a platform for the systemic uptake of *E. coli* O157:H7 to vascular and edible tissues of spinach plants.

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